The petroleum-degrading bacteria *Alcaligenes aquatilis* strain YGD 2906 as a potential source of lipopeptide biosurfactant.

Drifa Yalaoui-Guellal[a,b,*], Samira Fella-Temzi[b], Salima Djafri-Dib[c], Sunil Kumar Sahu[d], Victor U. Irorere[e], Ibrahim M. Banat[e] and Khodir Madani[f]

[a] Faculté des Sciences de la Nature et de la Vie et Sciences de la Terre, Université de Bouira, 10000 Bouira, Algérie.

[b] Laboratoire de Biomathématiques, Biophysique, Biochimie et Scientométrie (L3BS), Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie.

[c] Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie.

[d] State Key Laboratory of Biocontrol, Guangdong Key Laboratory of Plant Resource, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, P. R. China

[e] School of Biomedical Sciences, University of Ulster, Coleraine, County Londonderry, Northern Ireland, UK.

[f] Centre de Recherche en Technologies Agro-Alimentaires, Route Targua Ouzemmour, 06000 Bejaia, Algérie.

[(*)] Corresponding author. Tel.: +21334813710; fax: +21334813710; E-mail address: d.guelal@univ-bouira.dz

Abstract

Soummam river sediments were used to isolate a biosurfactant-producing and petroleum-degrading bacterium. This strain was identified as *Alcaligenes aquatilis* YGD 2906 using phenotypic characterization and 16S ribosomal RNA sequencing. The culture supernatant of the isolated strain showed no haemolytic activity had an oiled displacement of 23.66 ± 0.57 mm and Emulsification index (E24) of 68.5% ± 0.5%. The biosurfactant produced in minimal medium was extracted by acid precipitation and quantified gravimetrically, with a yield of 4.2 ± 0.01 g / L. The crude Biosurfactant was determinate by TLC and MALDI-TOF-MS as a lipopeptide with protein and lipid content of8.49 ± 0.19% and 52.66 ± 1.16% respectively. This lipopeptide structure was confirmed by HPLC-MS/MS. This technique gave two main peak ranges which are typical of surfactins, iturins and fengycin. Tandem mass spectrometry was further used to elucidate the structure of the lipopeptide produced by the strain. The non-haemolytic nature of the lipopeptide produced by this strain offers opportunities for biomedical applications. Further work is needed to optimize production and analyze potential biomedical uses of this lipopeptide including antimicrobial properties.

Keywords: Petroleum biodegradation, *Alcaligenes aquatilis* strain YGD 2906, Biosurfactant production, MALDI-TOF/MS analysis, HPLC-MS/MS.

1. Introduction

Microorganisms capable of degrading hydrophobic substances such as petroleum, are often able to produce natural surfactants or biosurfactants, which are surface-active agents produced by bacteria, yeast and filamentous fungi. These amphiphilic molecules with both hydrophobic and hydrophilic fragments can demonstrate various surface activities, which among other roles, can help to disperse and stabilise hydrophobic substances such as petroleum in water to form an emulsion. The microbial origin and the chemical composition are generally used to classify these biosurfactants which are mainly glycolipids, lipopeptides, lipoproteins, neutral lipids, fatty acids and phospholipids molecules [1].

As biosurfactant are typically capable of emulsifying the hydrophobic (oil or hydrocarbon) phase, it allows their easier dispersion in water, thus increasing microbial access to these hydrophobic compounds and their availability to biodegradation. They also increase oil mobilization and extraction from rock formation, a process that can be used for cost-effective solutions to enhance oil recovery technologies [2]. Recently, interest in the novel and natural surfactant has increased, mainly due to their biodegradability and low toxicity [3].

Although the microbial bioremediation of petroleum hydrocarbon is well known [4], these recalcitrant contaminants have limited bioavailability in aqueous systems, thus hindering their utilisation or biodegradation by existing microbes [5]. Therefore, the use of biosurfactant producing strains that can degrade petroleum hydrocarbon, does offer the benefit of an accelerated biodegradation process by increasing their bioavailability [5, 6]. However, while the benefits offered by biosurfactant producers in petroleum hydrocarbon

degradation is well known, studies aimed at characterising biosurfactants in such processes are scarce [7].

Thus, the aims of this study were to isolate petroleum degrading bacteria from Soummam river sediments, investigate their biosurfactant production capacity and fully characterise the biosurfactant produced using different methodologies such as TLC, FTIR, MALDI-TOF and HPLC-MS/MS.

2. Material and methods

2.1. Isolation of petroleum-degrading bacteria

Petroleum-degrading bacteria enrichment and isolation were carried out according to the method reported by [8]. In brief, 10gfresh sediments from Soummam river Skala Bridge in Bejaia, Algeria, which was contaminated with different petroleum derivates, was added to a 500 ml flat bottle containing 100 ml physiological saline (1% NaCl) and subjected to vigorous agitation for 1–2 h and let stand for 1 h afterwards. Five ml of the supernatant were transferred into Erlenmeyer flasks containing 45 ml Mineral Salt Medium (MSM),containing 3.0 g/L of Na_2HPO_4, 1.0 g/L of NH_4NO_3, 30 g/L of NaCl, 0.7 g/L of $MgSO_4.7H_2O$, 2.0 g/L of KH_2PO_4 and 1.0 ml of trace element solution. The trace element solution contained: 10 mg/L of $ZnSO_4.7H2O$, 0.50 mg/L of $CuSO_4.5H_2O$, 0.50 mg/L of $MnSO_4.H_2O$, 20 mg/L of $CaCl_2$ and30 mg/L of $FeCl_3$.This culture medium was supplemented by 2% (v/v) of crude petroleum as an energy and carbon source to enrich petroleum-degrading microbial strains [8]. Afterward, flasks were incubated at 30 °C on a shaker at 150 rpm for 7 days. Five ml of culture medium were transferred to fresh petroleum + MSMmix and incubated at the same conditions to obtain petroleum degrading enriched consortium, this process was carried out for four times. The isolation

of pure bacterial strains was carried out by spreading onto petroleum coated MSM agar plates and incubated at 37°C for 3-5 days. The morphologically distinct colonies were maintained on nutrient agar plates at 4°C.

2.2. Biosurfactant production test

The detection of biosurfactant production by bacterial isolate was carried out using three screening techniques including haemolytic activity in 5% blood agar plate (the ability to hydrolyze blood cells in blood agar) [9], oil displacement test (the ability to form a clear zone) [10, 11] and emulsification activity (The ability to emulsify hydrocarbons) [12]. All experiments were carried out in triplicates.

2.3. Phenotypical identification of petroleum-degrading bacterial isolates

Phenotypical identification of petroleum-degrading bacteria was carried out using Gram staining, citrate utilization, oxidase production, catalase test, methyl red, Vogues Proskauer test, gelatine liquefaction test, triple sugar iron test, fermentation of carbohydrate, urease test, indole production test and nitrate reduction [8].

2.4. 16S ribosomal DNA (rDNA) sequencing of bacterial isolates

The genetic identification of the isolated bacteria was carried out by 16S ribosomal DNA (rDNA) sequencing using universal primers 27F (5'-AGAGATTGATCCTGGCTCT G-3') and 1492R (5'-GGTTTCCTTGTTACGAC AT-3'). After the comparison between the obtained specific sequences and known 16S rDNA sequences, the sequences were further explored using nucleotide BLAST of NCBI database [13].

2.5. Biosurfactant production and extraction

111 A single colony of the bacterial strains were transferred into a 100 ml Erlenmeyer flask
112 containing 25 ml MSM broth supplemented with2% (v/v) of glucose and 0.1 g/L of yeast
113 extract, followed by incubation at 30°C under 150 rpm agitation for 24 h. Afterward, 5ml
114 of preculture content was used to inoculate 500ml of production medium Erlenmeyer
115 flask and incubated at 30°C on a rotary shaker at 150 rpm for 96h. Samples were then
116 centrifuged (12,000 g at 4°C for 20 min) to remove the bacterial cells. The 6N HCl acid
117 was then used to precipitate the cell-free supernatants to pH 2.0 - 3.0. Subsequently, the
118 precipitate was collected by centrifugation and the biosurfactant solution was freeze-dried
119 [8].

120 **2.6 Characterization of biosurfactants**

121 **2.6.1. Thin layer chromatography analysis**

122 A small amount of the biosurfactants extract was dissolved in chloroform, and analyzed
123 by Thin layer chromatography (TLC) using silica gel 60 coated glass sheet (Silica gel
124 $60F_{254}$, MERCK, Germany). The plates were developed using solvent mixture:
125 chloroform-methanol-water (65/15/4; v/v/v). Spots were revealed by spraying with
126 solution of ninhydrin for the detection of compounds with free amino groups, after
127 heating at 110 °C for 5 min [14].

128 **2.6.2. Chemical composition of biosurfactant**

129 The method of Morikawa and coworkers [15] was used to evaluate the lipid content of
130 the biosurfactants. In brief 10 mg of freeze-dried biosurfactant were extracted with
131 mixture of chloroform: methanol: water in the ratios 2: 2: 0.8 (v / v / v). The collected
132 chloroform phase containing the purified lipids was evaporated at 40-60°C for 20 min.
133 The content of residual lipid was determined gravimetrically.

Total protein content of the biosurfactants was determined according to the Bradford method [16]. Briefly,1 ml of Bradford reagent was mixed with 20 µl of the standard gold sample, and the mixture was well shaken. Absorbance was measured at 595 nm and the concentration of protein was monitored using the calibration curve with Bovine Serum Albumin as standard [17].

2.6.3. Fourier Transform Infrared Spectroscopy characterization

Fourier transform infrared spectroscopy (FTIR) using the potassium bromide (KBr) pellet method was used to identify the functional group in the biosurfactant sample.0.2 mg biosurfactant samples were mixed with 90 mg of dry KBr to form a very fine powder. This powder was compressed to obtain translucent pellet which could be analyzed by the FTIR system (IRaffinity-1, SHIMADZU). The spectrum was obtained in the range of 400–4000 cm^{-1}. Then, basic functional groups of the biosurfactant were analyzed according to [17].

2.6.4. Molecular mass determination by MALDI-TOF-MS

Initial analysis was carried out by MALDI-TOF-MS. A Perspective Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK) with a 1 m time-of-flight tube was used for the analysis. The instrument was first calibrated internally using a peptide calibration mix consisting of 4 peptides. A concentration of 1mg/ml was obtained after reconstitution and dilution of the sample of biosurfactants in methanol. Dihydroxyl benzoic acid was used as the matrix solution [18] and a mass range of 400 Da to 2000Da was selected, as most known lipopeptides are within this range [19-21].

2.6.5. HPLC-MS/MS analysis

156 HPLC-MS method used is as reported previously [22]. The analysis was performed on an
157 LC P400 (Thermofiningan) in line with an LCQ quadruple ion trap mass spectrometer
158 (Thermofiningan) equipped with an ESI-source and operated in a positive ionization
159 mode. An Agilent Poroshell 120, EC-C18, 2.1 ×100 mm, 2.7µm was used as a static phase
160 while the mobile phase consisted of 1% formic acid in water and acetonitrile, respectively.
161 The gradient used is as described by [22].

162 **2.7. Statistical analysis**

163 The experimental data were presented as averages of three replicates, Standard deviations
164 were represented with error bars.

165 **3. Results and Discussion**

166 **3.1. Screening of Biosurfactant production**

167 In this study, we have used the enrichment technique, with mineral salt medium (MSM),
168 to isolate a petroleum-degrading bacteria from the Soummam river sediments in Algeria
169 .As this sediment has been the subject of several hydrocarbon contamination in recent
170 years, microorganisms there have adapted to utilising petroleum hydrocarbon as their sole
171 carbon and energy sources[8].

172 Divers tests have been identified for the screening of potential biosurfactant producing
173 isolates. These tests have been extensively reviewed by various authors and include the
174 following: haemolytic assay, surface/interfacial assay, emulsification assay/index and oil
175 displacement assay [23-25]. Thus, using a number of these screening methodologies, the
176 potential biosurfactant production by the isolated strain was investigated.

First, no haemolysis was observed using the haemolytic activity test of the culture supernatant. While haemolysis is an indication of biosurfactant production, it is not precise and can give a lot of false negative or positive results [23]. Indeed, some biosurfactant has been reported to lack haemolytic activity all together [26]. Das and co-workers [27] showed the production and antimicrobial activity of non-haemolytic lipopeptide species from a marine *Bacillus circulans* strain, noted that nonhaemolytic biosurfactants holds huge potential for biomedical applications. The authors further recommended the safe use of purified biotensio-active lipopeptide product as a potential antimicrobial candidate in humans and animals. This result is particularly useful as most potent biosurfactants with antimicrobial properties also possess haemolytic activities, thus disqualifying them as potential drugs for humans and animals [28].

The oil displacement test measures the diameter of the clear zone formed by the surface activity of the surfactant solution tested, with respect to the oil. A high surface activity of the biosurfactant is indicated by a large diameter of the oil displacement. The supernatant of the isolated strain gave a diameter of 23.66 ± 0.57 mm, which indicates the presence of high concentrations of surface-active compounds. The microbial production of surfactants by microorganisms growing on crude oil and other hydrophobic substances has been frequently reported [29, 30]. Furthermore, the emulsification index (E_{24}) of the culture supernatant for the isolated strain was found to be very interesting. The value of the emulsification index (E_{24}) was 68.5±0.5%, this result was higher than the positive control (SDS) (64.77±1.6%). The crude oil emulsification in water is a prerequisite which opens the way for the biodegradation of this environmental pollutant by many bacteria. It improves the bioavailability of petroleum and thus increases the rate of biodegradation [31-33].

3.2. Identification of the isolate

The petroleum degrading bacterial isolate was identified as *Alcaligenes aquatilis* strain YGD2906, according to 16S rDNA gene sequences and Neighbor-joining phylogenetic analysis (Fig. 1). This strain was confirmed as gram-negative, while oxidase, catalase and urease production tests were positive. All other tests such as citrate utilization, methyl red, Vogues Proskauer test, gelatine liquefaction test, triple sugar iron test, fermentation of carbohydrate, indole production test and nitrate reduction were negative.

The evolutionary history was inferred using the Neighbour-Joining method [34]. The optimal tree with the sum of branch length = 0.06473377 is shown. The percentage of bootstrap value (1000 replicates) is shown next to the branches [35]. The evolutionary distances were computed using the p-distance method [36] and are in the units of the number of base differences per site. Among the eight nucleotide sequences, the positions containing gaps and missing data were excluded from the analysis, resulting in a total of 1371 positions. MEGA7 was used for the evolutionary analyses [37]. The sequence is deposited in Genbank under accession number MT103125.

3.3. Biosurfactant production, extraction and quantification

Production of biosurfactant by the strain *Alcaligenes aquatilis* YGD2906 was carried out in MSM broth supplemented with 2% (v/v) glucose and 0.1 g/L of yeast extract. Cells were incubated at 30°C with stirring at 150 rpm for 96 h. After extraction by acid precipitation, a total of 4.2± 0.01 g/L crude freeze-dried biosurfactant was obtained. The value of the emulsification index (E_{24}%) of this biosurfactant was 73.23 ± 1.61%, which was higher than the positive control (SDS) (64.77 ± 1.6%).However, this quantity is quite appreciable compared to that observed by others [38, 39].

3.4. Characterization of biosurfactant produced by *Alcaligenes aquatilis* YGD2906

3.4.1. Thin layer chromatography (TLC) analysis and Chemical composition of biosurfactant

TLC spots were visible on plates after spraying with ninhydrin reagent, indicating the presence of free amino acids. These results suggest the lipopeptide nature of the biosurfactant produced by the isolated strain, similar to those reported previously for other bacterial strains [27, 40, 41]. The lipopeptide nature of the biosurfactant was further confirmed by protein and lipid content determination, which was 8.49 ± 0.19% and 52.66 ± 1.16% respectively. These results confirm the lipopeptide nature of the biosurfactant produced by the isolated *Alcaligenes aquatilis* YGD 2906.

3.4.2. Fourier Transform Infrared Spectroscopy (FTIR) Characterization

The functional groups present in the produced biosurfactant were identified by FTIR analysis. The FTIR spectrum (Fig. 2), illustrates the presence of both an aliphatic and peptide chain.

The peaks at 3419 $cm^{-1}$and 1657 cm^{-1}corresponded to stretching and bending of N–H group, respectively. The peaks at 2924 and 2862 cm^{-1} indicated the presence of methyl and methylene, respectively. The peaks at 1722, 1443, 1383, 1233 cm^{-1} showed the stretching of C=O, aliphatic chain (–CH2−), C–H bend, acyl and phenyl C–O, respectively. A peak at 1072 cm^{-1} pointed out the presence of alkoxy group. FTIR results, therefore, indicated the presence of aliphatic hydrocarbon in combination with peptide group, which is the characteristic feature of lipopeptide biosurfactants such as surfactin [42, 43].

3.4.3. Molecular mass determination by MALDI-TOF-MS

The results from the MALDI-TOF-MS are presented in Fig.3a. Two main peak ranges were identified: 1008 to 1105 and 1450 to 1585. These peaks are typical of Surfactins, Iturins and Fengycins. The major peaks within these range and their potential lipopeptide assignments are shown in Table 1 [44]. The Table shows mass structures 1047,1061 and 1075 typical for surfactins containing 13,14 and 15 carbon atoms, respectively. It also shows mass structures 1464, 1478,1493, and 1505 which we believe represents different Fengycin type surfactants based on their MS/MS analysis which generated molecular structure breakdown expected for these type of molecules as detailed below.

3.4.4. HPLC-MS/MS analysis

Following results from the MALDI-TOF-MS, further characterization was carried out using HPLC-MS to separate the individual species. The full ESI-MS is presented in Fig.3b and showed 2 distinct peak patterns as those observed in MALDI-TOF/MS with additional peaks at 751 to 773 corresponding to the double charged 1464 to 1506 [45] having a proposed K^+. The results from HPLC-MS are presented in Table 2. Other peaks were also identified but these have a series of contaminating fractions that could not be resolved and hence are not presented in Table 2.

To further characterize the lipopeptides, another HPLC-MS run was carried out with an added setting to characterise all relevant peaks (Fig. 4) by MS/MS and further characterise relevant MS/MS products by MS3 with dissociation energy of 35. At the collision energy used, only peaks 1464, 1478, 1492 and 1506 gave values that can be further characterized (Fig.5). These peaks were initially thought to be C16 to C19 Fengycin A $(M+H)^+$, respectively. However, the collision induced dissociation (CID) showed that 1464 and

1478 has daughter ions at 966 and 1080 while 1492 and 1506 has daughter ions at 994 and 1108. The latter are indicative of C16 and C17 Fengycin A $(M+H)^+$with D-Ala at position 6 while the former is indicative of C16 and C17 Fengycin B $(M+H)^+$with D-Val at position 6 (Fig.6a and b) [22].

4. Conclusion

This study was the first research about introducing *Alcaligenes aquatilis* YGD 2906 as a petroleum-degrading bacteria strain, capable of biosurfactant production. Different methods such as TLC, FTIR, MALDI-TOF/MS were used to characterize the biosurfactant produced by this strain. The results showed that this strain can produce several lipopeptide species particularly Surfactins and Fengycins. Following chromatographic separation, MS/MS analysis was used to resolve the chemical structure of Fengycin A and B, produced by the isolated strain. The produced lipopeptide also showed no haemolytic activities, thus making it a desirable candidate for biomedical applications, including use as antimicrobial agents as previously suggested for non-haemolytic lipopeptides by [27].

Acknowledgments

A grant from The Ministry of High Education and Scientific Research of Algeria was supported this study. All authors thank everyone who contributed to the realization of this work. We would like to thank Penedo P.M. at laboratory StabVIDA (Portugal) for molecular identification of species study.

References

290 [1] Silva RCFS, Almeida DG, Rufino RD, Luna JM, Santos VA, Sarubbo LA.
291 Applications of biosurfactants in the petroleum industry and the remediation of oil
292 spills. International Journal of Molecular Sciences. 2014;15: 12523–12542.

293 [2] Ibrahim ML, Ijah UJJ, Manga SB, Bilbis LS, Umar S. Production and partial
294 characterization of biosurfactant produced by crude oil degrading
295 bacteria. International Biodeterioration & Biodegradation. 2013; 81: 28-34.

296 [3] Irorere VU, Tripathi L, Marchant R, McClean S, Banat IM. Microbial rhamnolipid
297 production: a critical re-evaluation of published data and suggested future
298 publication criteria. Applied Microbiology and Biotechnology. 2017; 101(10):
299 3941-3951.

300 [4] Xu X, Liu W, Tian S, Wang W, Qi Q, Jiang P, Gao X, Li F, Li H, Yu H. Petroleum
301 hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic
302 conditions: a perspective analysis. Frontiers in microbiology. 2018; 3(9):2885.

303 [5] Patowary K, Patowary R, Kalita MC, Deka S. Characterization of biosurfactant
304 produced during degradation of hydrocarbons using crude oil as sole source of
305 carbon. Frontiers in microbiology. 2017; 22(8):279.

306 [6] Das N, Chandran P. Microbial degradation of petroleum hydrocarbon contaminants:
307 an overview. Biotechnology research international. 2011;2011.

308 [7] Chandankere R, Yao J, Cai M, Masakorala K, Jain AK, Choi MM. Properties and
309 characterization of biosurfactant in crude oil biodegradation by bacterium *Bacillus*
310 *methylotrophicus* USTBa. Fuel. 2014;122:140-8.

311 [8] Yalaoui-Guellal D, Brahmi F, Touati A, De Champs C, Banat IM, Madani K.
312 Production of Biosurfactants by Hydrocarbons Degrading Bacteria Isolated from
313 Soummam Watershed Sediments of Bejaia in Algeria. Environmental Progress &
314 Sustainable Energy. 2018;37(1):189-1950.

315 [9] Hassanshahian M. Isolation and characterization of biosurfactant producing bacteria
316 from Persian Gulf (Bushehr provenance). Marine pollution bulletin. 2014; 86(1):
317 361-366.

318 [10] Morikawa M, Hirata Y, Imanaka T.A study on the structure–function relationship of
319 lipopeptide biosurfactants, Biochimica et Biophysica Acta (BBA)-Molecular and
320 Cell Biology of Lipids. 2000; 1488: 211–218.

321 [11] Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, Mc Inerney MJ.
322 Comparison of methods to detect biosurfactant production by diverse
323 microorganisms, Journal of Microbiological Methods.2004; 56:339–347.

324 [12] Emtiazi G, Saleh T, Hassanshahian M. The effect of bacterial glutathione S-
325 transferase on morpholine degradation, Biotechnology Journal.2009; 4: 202–205.

326 [13] Fella-Temzi S, Yalaoui-Guellal D, Rodriguez-Carvajal MA, Belhadi D, MadaniK,
327 Kaci Y. Removal of lead by exopolysaccharides from *Paenibacillus peoriae*
328 strainTS7 isolated from rhizosphere of durum wheat. Biocatalysis and agricultural
329 biotechnology. 2018; 16: 425-432.

330 [14] Noparat P, Maneerat S, Saimmai A. Utilization of palm oil decanter cake as a novel
331 substrate for biosurfactant production from a new and promising strain of

Ochrobactrum anthropi 2/3. World Journal of Microbiology and Biotechnology. 2014; 30(3): 865-877.

[15] Bligh EG, Dyer WJ.A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology.1959; 37(8): 911-917.

[16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry.1976; 72(1-2): 248-254.

[17] Yalaoui-Guellal D, Fella-Temzi S, Djafri-Dib S, Brahmi F, Banat IM, Madani K. Biodegradation potential of crude petroleum by hydrocarbonoclastic bacteria isolated from Soummam wadi sediment and chemical-biological proprieties of their biosurfactants. Journal of Petroleum Science and Engineering. 2020;184: 106554.

[18] Teearu A, Vahur S, Haljasorg U, Leito I, Haljasorg T, Toom L. 2, 5-Dihydroxybenzoic acid solution in MALDI-MS: ageing and use for mass calibration. Journal of Mass Spectrometry. 2014; 49(10): 970-979.

[19] Smyth T, Perfumo A, McClean S, Marchant R, Banat I. Isolation and Analysis of Lipopeptides and High Molecular Weight Biosurfactants. In Handbook of hydrocarbon and lipid microbiology. 2010; 3688-3704.

[20] Meena KR, Sharma A, Kanwar SS. Microbial lipopeptides and their medical applications. Annals of Pharmacology and Pharmaceutics. 2017; 2 (21): 1111.

[21] Zhang L, Sun C. Fengycins, cyclic lipopeptides from marine Bacillus subtilis strains, kill the plant-pathogenic fungus *Magnaporthe grisea* by inducing reactive oxygen

353 species production and chromatin condensation. Applied and Environmental
354 Microbiology. 2018; 84(18): e00445-18.

355 [22] Pecci Y, Rivardo F, Martinotti MG, Allegrone G.LC/ESI-MS/MS characterisation
356 of lipopeptide biosurfactants produced by the *Bacillus licheniformis* V9T14 strain
357 Journal of Mass Spectrometry.2010; 45:772-778.

358 [23] Walter V, Syldatk C, Hausmann R. Screening concepts for the isolation of
359 biosurfactant producing microorganisms. In Biosurfactants.2010; 1-13.

360 [24] Satpute SK, Banpurkar AG, Dhakephalkar PK, Banat IM, Chopade BA. Methods for
361 investigating biosurfactants and bioemulsifiers: a review. Critical reviews in
362 biotechnology. 2010; 30(2): 127-144.

363 [25] Varjani SJ, Upasani VN. Critical review on biosurfactant analysis, purification and
364 characterization using rhamnolipid as a model biosurfactant. Bioresource
365 technology. 2017; 232: 389-397.

366 [26] Schulz D, Passeri A, Schmidt M, Lang S, Wagner F, Wray V, Poremba K, Gunkel
367 W. Screening for biosurfactants among crude oil degrading marine
368 microorganisms. Kieler Meeres for schungen. Sonderheft. 1991.

369 [27] Das P, Mukherjee S, Sen R. Antimicrobial potentials of a lipopeptide biosurfactant
370 derived from a marine *Bacillus circulans.* Journal of Applied Microbiology. 2008;
371 104(6):1675-1684.

372 [28] Symmank H, Franke P, Saenger W, Bernhard F. Modification of biologically active
373 peptides: production of a novel lipohexapeptide after engineering of *Bacillus*
374 *subtilis* surfactin synthetase. Protein engineering. 2002; 15(11): 913-921.

375 [29] Iqbal S, Khalid Z, Malik K. Enhanced biodegradation and emulsification of crude
376 oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of
377 *Pseudomonas aeruginosa*. Letters in Applied Microbiology.1995;21(3): 176-179.

378 [30] Kumar M, Leon V, Materano ADS, Ilzins OA, Galindo-Castro I, Fuenmayor SL.
379 Polycyclic aromatic hydrocarbon degradation by biosurfactant-producing
380 *Pseudomonas sp*. IR1. Zeitschriftfür Natur for schung C. 2006; 61(3-4): 203-212.

381 [31] Quek E, Ting YP, Tan HM. *Rhodococcus sp*. F92 immobilized on polyurethane foam
382 shows ability to degrade various petroleum products. Bioresource Technology.
383 2006; 97(1): 32-38.

384 [32] Bredholt H, Josefsen K, Vatland A, Bruheim P, Eimhjellen K. Emulsification of
385 crude oil by an alkane-oxidizing Rhodococcus species isolated from seawater.
386 Canadian Journal of Microbiology. 1998; 44(4): 330-340.

387 [33] Mnif S, Chamkha M, Labat M, Sayadi S. Simultaneous hydrocarbon biodegradation
388 and biosurfactant production by oilfield selected bacteria. Journal of Applied
389 Microbiology. 2011; 111(3): 525-536.

390 [34] Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing
391 phylogenetic trees. Molecular Biology and Evolution.1987; 4: 406-425.

392 [35] Felsenstein J, Confidence limits on phylogenies: An approach using the bootstrap.
393 Evolution. 1985;39:783-791.

394 [36] Nei M, Kuma, S. Molecular Evolution and Phylogenetics. Oxford University Press,
395 New York. 2000.

396 [37] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis
397 version 7.0 for bigger datasets. Molecular Biology and Evolution.2016; 33:1870-
398 1874.

399 [38] Maalej H, Hmidet N, Boisset C, Bayma E, Heyraud A, Nasri M. Rheological and
400 emulsifying properties of a gel-like exopolysaccharide produced by *Pseudomonas*
401 *stutzeri* AS22. Food Hydrocolloids.2016; 52: 634-647.

402 [39] Kuyukina MS, Ivshina IB. Multifunctional biosurfactant from non-pathogenic
403 *Rhodococcus ruber* for diverse industrial applications. Journal of
404 Biotechnology.2010; 150: 83-84

405 [40] Sriram MI, Kalishwaralal K, Deepak V, Gracerosepat R, Srisakthi K, Gurunathan S.
406 Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced
407 by heavy metal tolerant strain *Bacillus cereus* NK1, Colloids and Surfaces B:
408 Biointerfaces. 2011; 85(2): 174-181.

409 [41] Bezza FA, Chirwa EMN. Production and applications of lipopeptide biosurfactant
410 for bioremediation and oil recovery by *Bacillus subtilis* CN2. Biochemical
411 Engineering Journal.2015; 101: 168-178.

412 [42] Liu B, Liu J, Ju M, Li X, Yu Q. Purification and characterization of biosurfactant
413 produced by *Bacillus licheniformis* Y-1 and its application in remediation of
414 petroleum contaminated soil. Marine Pollution. Bulletin. 2016; 107 (1): 46–51.

415 [43] Pereira JF, Gudiña EJ, Costa R, Vitorino R, Teixeira JA, Coutinho JA, Rodrigues
416 LR. Optimization and characterization of biosurfactant production by *Bacillus*

417 *subtilis* isolates towards microbial enhanced oil recovery applications. Fuel. 2013;
418 111: 259–268.

419 [44] Dimkić I, Stanković S, Nišavić M, Petković M, Ristivojević P, Fira D, Berić T. The
420 Profile and Antimicrobial Activity of Bacillus Lipopeptide Extracts of Five
421 Potential Biocontrol Strains. Frontiers in Microbiology 8. 2017. Doi
422 : 10.3389/fmicb.2017.00925

423 [45] Ma Y, Kong Q, Qin C, Chen Y, Chen Y, Lv R, Zhou G. Identification of lipopeptides
424 in *Bacillus megaterium* by two-step ultrafiltration and LC–ESI–MS/MS. AMB
425 Express. 2016;6(1):79.

426

Table 1

Lipopeptide assignment from MALDI-TOF/MS

Mass	Lipopeptide assignment	References
1047.20	C13 Surfactin $(M+K)^+$	[44]
1061.31	C14 Surfactin $(M+K)^+$	proposed
1075.34	C15 Surfactin $(M+K)^+$	[44]
1464.45	C16 Fengycin A $(M+H)^+$	Based on MS/MS analysis
1478.73	C17 Fengycin A $(M+H)^+$	Based on MS/MS analysis
1493.03	C18 Fengycin B $(M+H)^+$	Based on MS/MS analysis
1505.98	C19 Fengycin B $(M+H)^+$	Based on MS/MS analysis

Table 2

Assignment of Lipopeptide from LC/MS and their individual relative abundance in accordance to their class

m/z	Retention time	Relative abundance	Lipopeptide assignment
994	36.52	36.53	C12 Surfactin $(M+H)^+$
1008	39.44	63.47	C13 Surfactin $(M+H)^+$
1464	15.05	14.84	C16 fengycin A $(M+H)^+$
1478	16.07	35.53	C17 fengycin A $(M+H)^+$
1492	15.79	20.13	C16 fengycin B $(M+H)^+$
1506	16.99	29.50	C17 fengycin B $(M+H)^+$

458

459

460 **Figure Captions**

461 **Fig.1.**Neighbour-joining phylogenetic tree showing the taxonomic position of
462 *Alcaligenes aquatilis* strain YGD 2906. *Paenalcaligenes suwonensis* strain ABC02-12
463 (NR 133804.1) was used as the out-group.

464 **Fig.2.** FT-IR spectrum of the biosurfactant produced by *Alcaligenes aquatilis* strain
465 YGD 2906

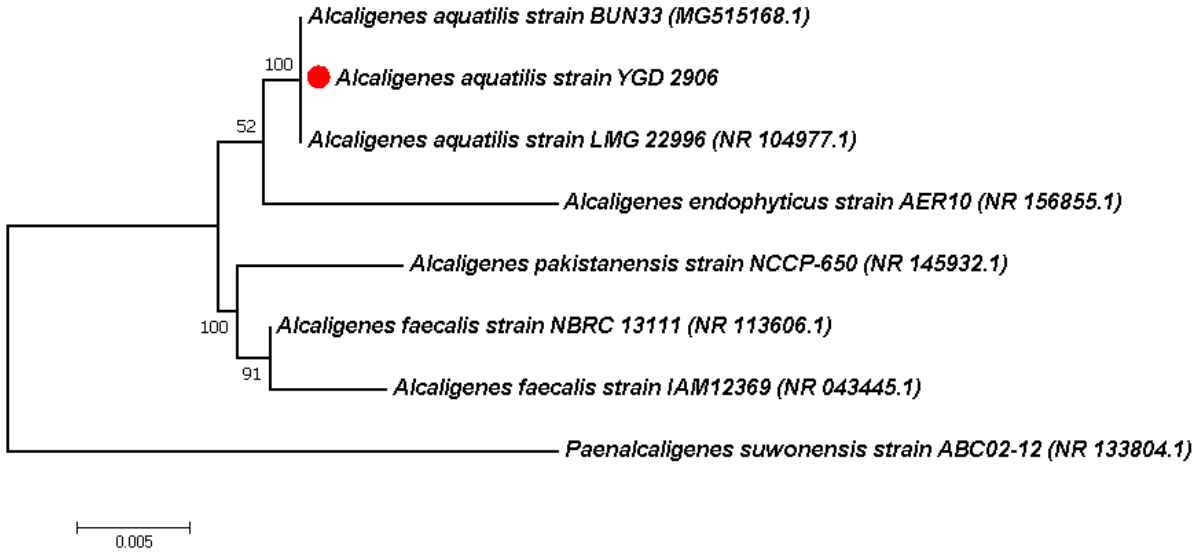
466 **Fig.3.** The a) MALDI-TOF/MS and b) ESI-full MS spectra of extracted lipopeptide,
467 showing two different peak ranges at 1008 to 1105 and 1450 to 1585. ESI-full MS was
468 taken after chromatographic separation as explained in materials and methods and is
469 representative of the entire chromatogram.

470 **Fig.4.** Chromatogram of Lipopeptide sample, showing the major peaks chosen for tandem
471 mass spectrometric analysis.

472 **Fig.5.** HPLC-MS/MS analysis of ions 1464, 1478, 1492 and 1506

473 **Fig.6.** Structures of Fengycin A $(M+H)^+$(a) and Fengycin B $(M+H)^+$ (b) showing the
474 different daughter ions observed in the MS/MS

475



476

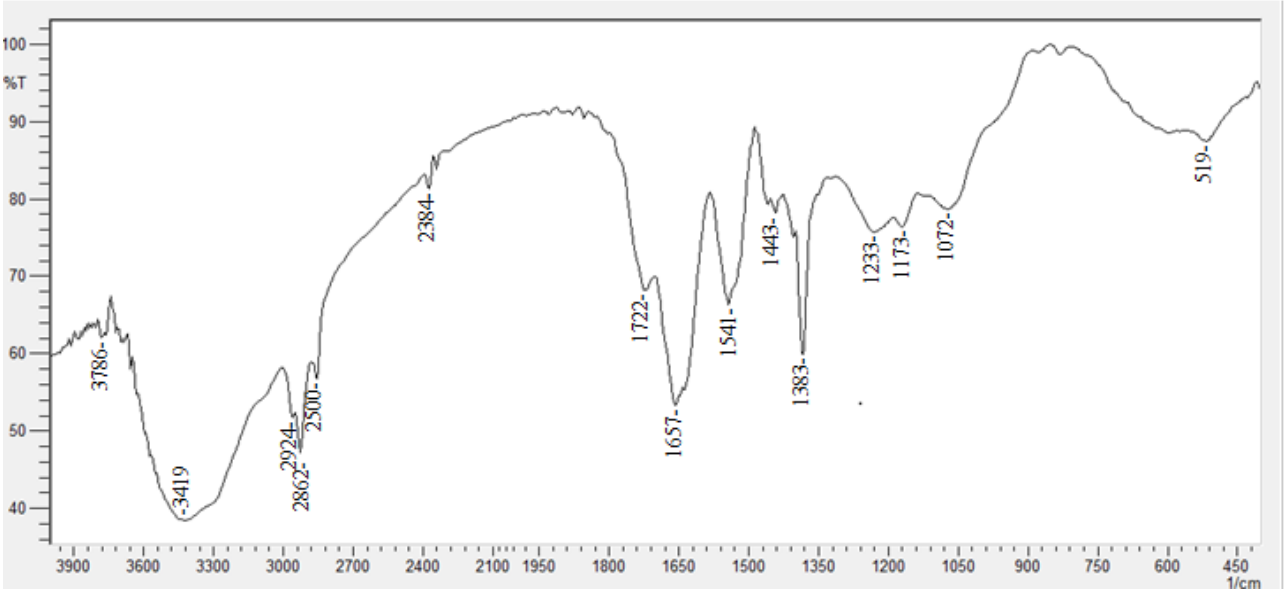
477 **Figure 1**: Neighbour-joining phylogenetic tree showing the taxonomic position of

478 *Alcaligenes aquatilis* strain YGD 2906. *Paenalcaligenes suwonensis* strain ABC02-12

479 (NR 133804.1) was used as the out-group.

480

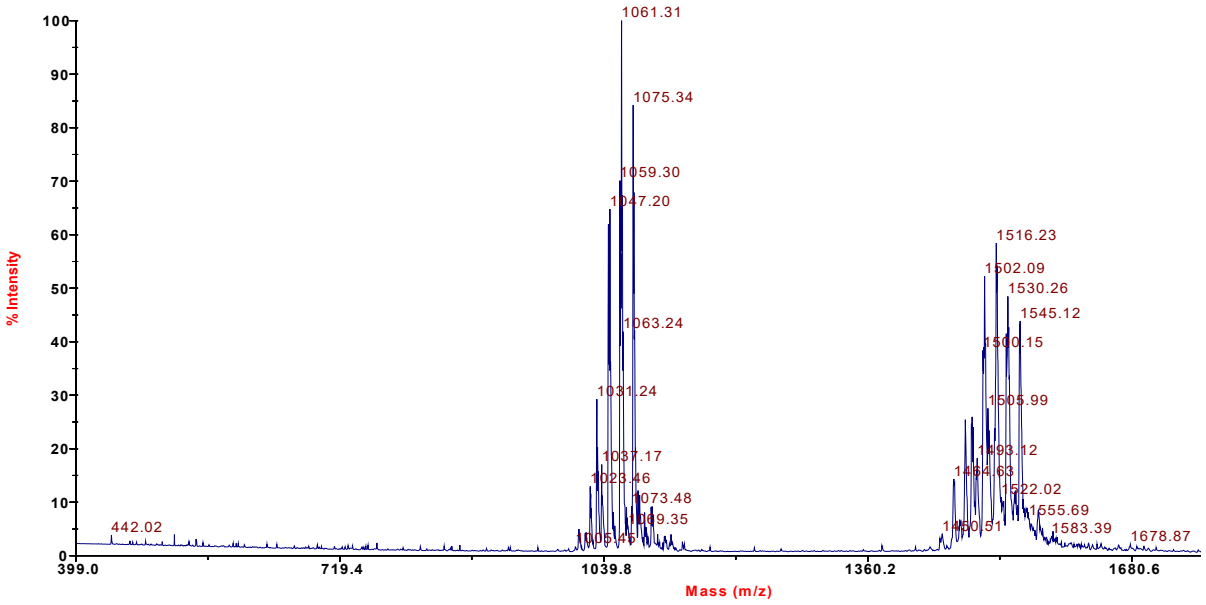
481



482

483 **Figure 2:** FT-IR spectrum of the biosurfactant produced by *Alcaligenes aquatilis* strain

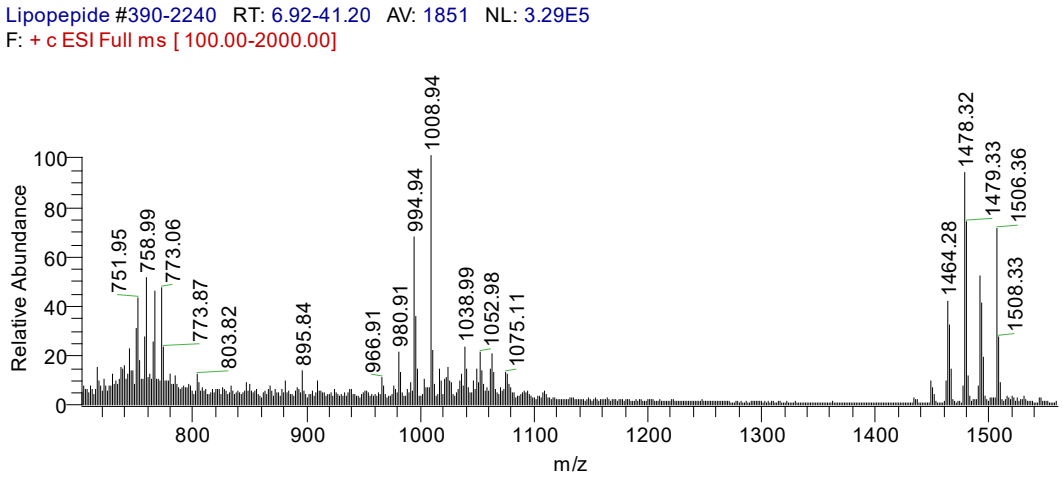
484 YGD 2906



488

489 **Figure 3:** MALDI-TOF/MS spectra of lipopeptide sample

490



491

492

493 **Figure 4:** ESI-full MS of lipopeptide sample from LC separation

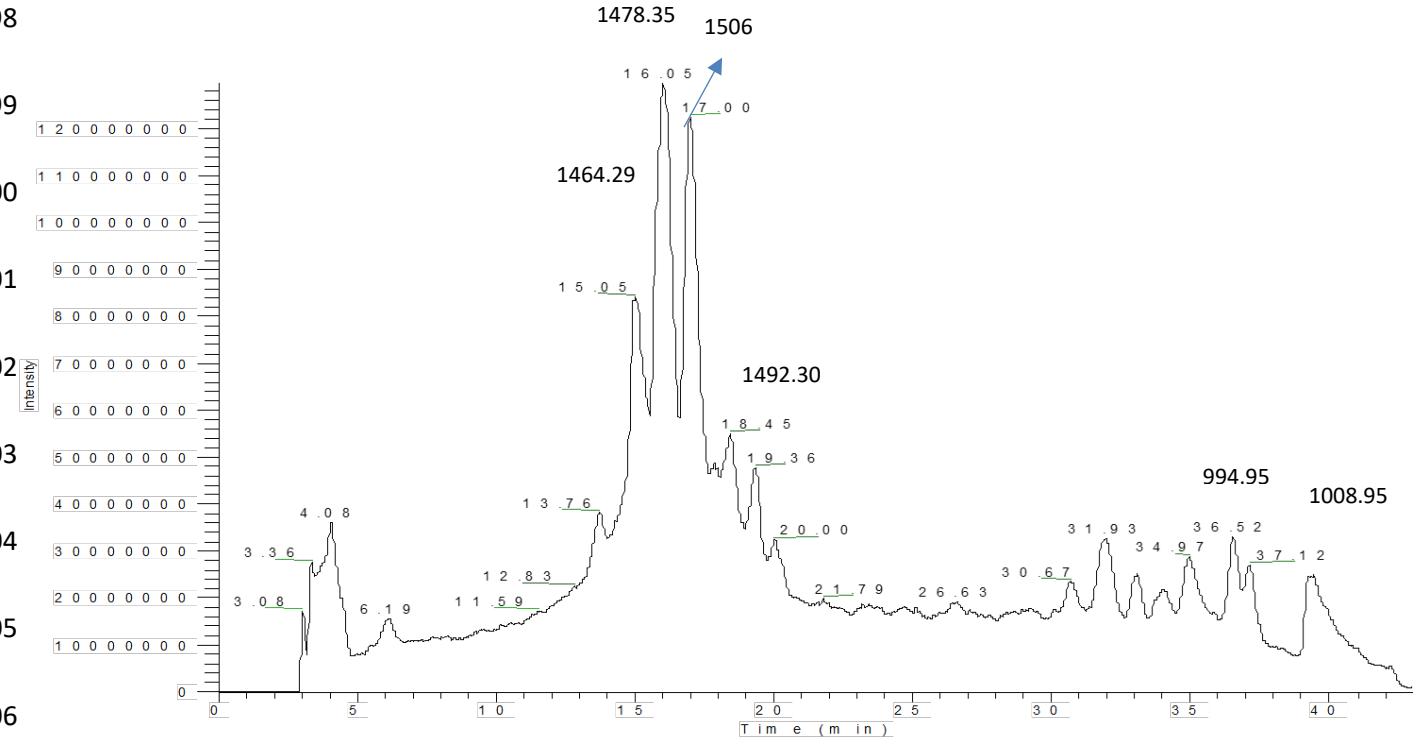


Figure 5: Chromatogram of Lipopeptide sample

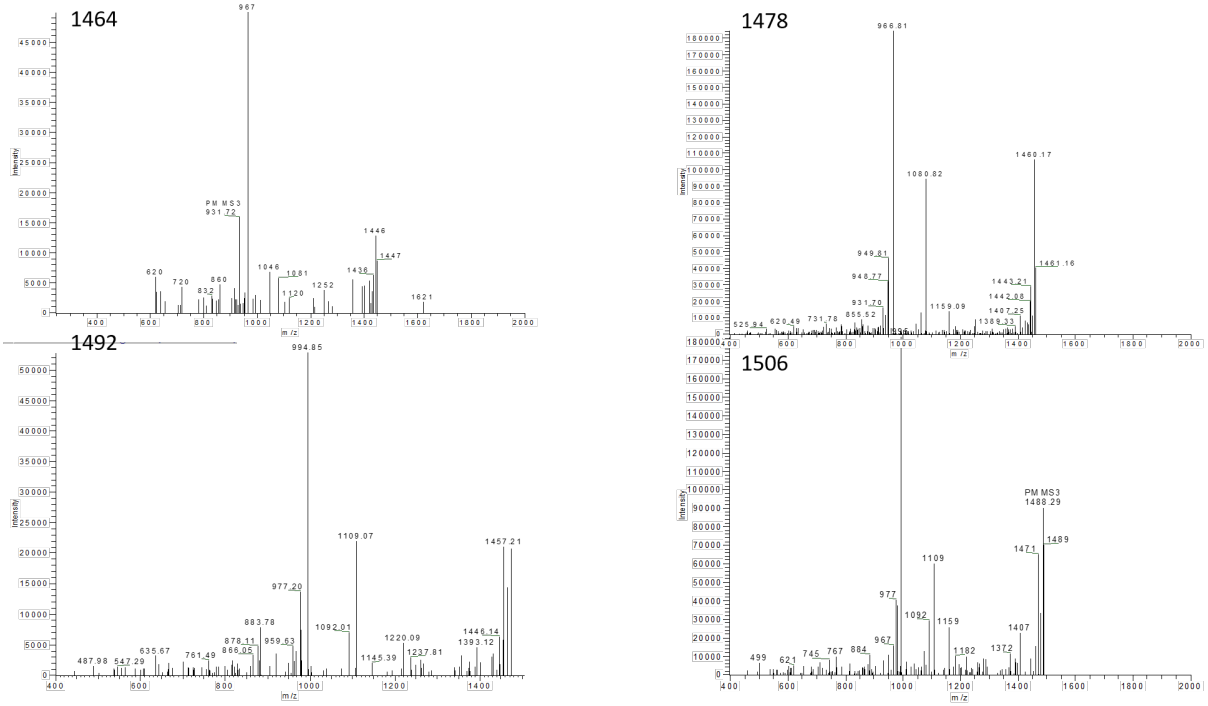


Figure 6: LC-MS/MS analysis of ions 1464, 1478, 1492 and 1506

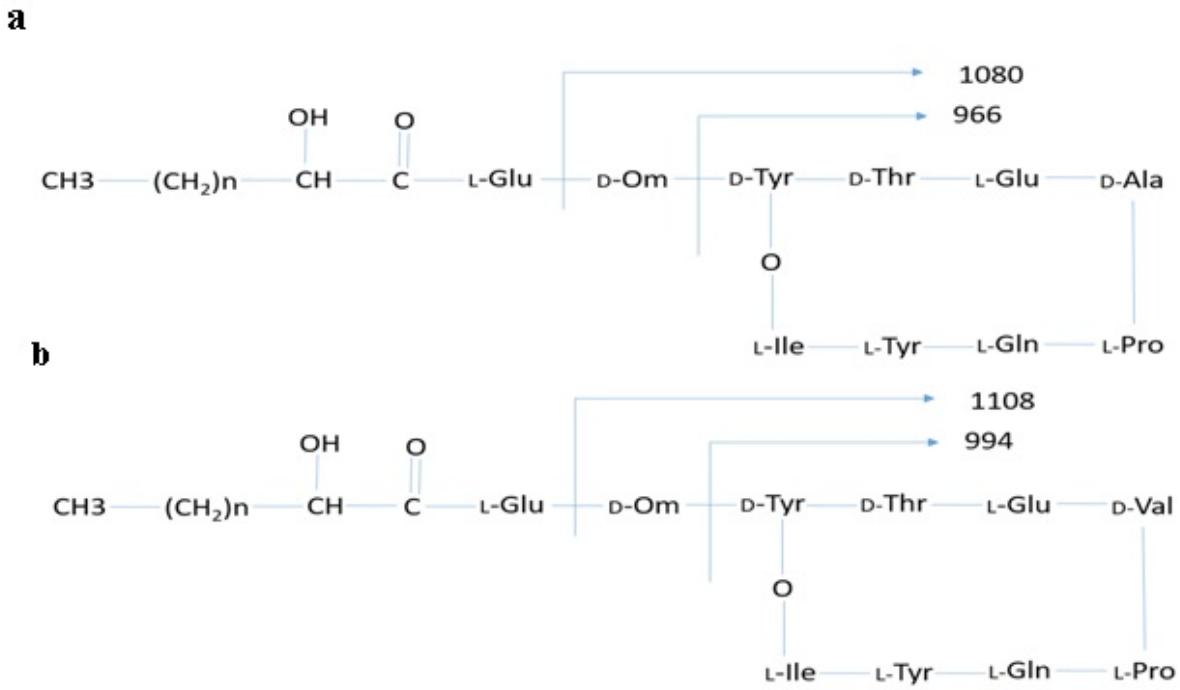


Figure 7: Structures of Fengycin A $(M+H)^+$(a) and Fengycin B $(M+H)^+$ (b) showing the different daughter ions observed in the MS/MS